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# NODs in defence: from vulnerable antimicrobial peptides to chronic inflammation

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**Defensins and cathelicidins are prevalent and essential gastrointestinal cationic antimicrobial peptides (CAPs). However, these defensive peptides are not infallible because certain enteropathogens can overcome their protective function. Furthermore, impaired defensin synthesis has been linked to the occurrence of Crohn's disease (CD), a chronic inflammatory bowel disease. Recently, defective bacterial sensing through NOD1 and NOD2 has been related to reduced defensin production, CD predisposition and susceptibility to enteric infection. Hence, we propose that microbial sensors at the gut interface monitor the levels of these effector peptides, which might function as 'danger' signals to confer tolerance and alert immunocytes. Further work is required to clarify how gastrointestinal CAPs are regulated and to assess their role in maintaining epithelial homeostasis and triggering adaptive immunity.**

## Linking inflammatory bowel disease to a vulnerable armoury of cytosolic innate sensors

The digestive mucosa has evolved various immune strategies to tolerate intimate contact with commensals and to prevent pathogenic bacteria from spreading into host tissues. The recognition of foodborne indigenous and pathogenic microbes is an essential barrier function for the survival of insects and mammals. In particular, mammalian resistance to pathogens is mainly conferred by membrane-bound Toll-like receptors (TLRs) [1] and the recently identified family of cytosolic nucleotide-binding oligomerization domain proteins that contain leucine-rich repeats (NOD-LRRs) [2]. NOD1 and NOD2 have an N-terminal caspase recruitment domain (CARD), a central nucleotide-binding domain (NOD) and a C-terminal leucine-rich repeat domain (LRR) [2].

Considerable attention has been focused on NOD2 signalling because mutations of this gene have been associated with Crohn's disease (CD), a nonspecific chronic inflammatory disease that can affect the whole human gastrointestinal tract [3,4]. NOD2 functions as a cytosolic pattern-recognition molecule (PRM) for bacterial peptidoglycan [5] by detecting a major muropeptide that is released and recycled during bacterial growth – the muramyl dipeptide MurNAc-L-Ala-D-isoGln (MDP) [6–8]. Following recognition of MDP, NOD2 promotes and regulates both innate and adaptive immunity through the activation of transcription factors and kinases [2]. Hence, the absence of Nod2 signalling in mice confers oral susceptibility to *Listeria monocytogenes* through the regulation of certain enteric cationic antimicrobial peptides (CAPs) [8]. Recent work has provided evidence that NOD1 confers responsiveness to peptidoglycans that contain meso-diaminopimelic acid (which is primarily found in Gram-negative bacteria) [9,10]. Similar to the physiological role of NOD2, NOD1 is required for expression of certain β-defensins by gastric epithelial cells during *Helicobacter pylori* infection [11].

In this Opinion article, we review recent findings on the physiological roles of NOD1 and NOD2 as sensors and on gut-derived CAPs (defensins and cathelicidins) as effectors of the gastrointestinal antimicrobial defence system (Figure 1 and Figure 2). We hypothesize that impaired microbial sensing and aggression by specific enteric microbes might tackle the function of CAPs in the gut and result in the development of chronic inflammatory disorders such as inflammatory bowel disease (IBD). These findings shed new light on the pathogenesis of CD, which is classically viewed as resulting from an abnormal T-cell activation by microbial immunogens.

## Gastrointestinal antimicrobial peptides: implications for inflammatory disease

Recent reports have shed light on the effector role of CAPs in monitoring gut homeostasis and in the containment of invading microbes because the stem cells that replenish the gut epithelium require continuous antimicrobial protection. The level of CAP expression parallels intestinal development in metazoans, from the immaturity of local defence mechanisms during gestation to bacterial colonization of the gut after birth [12]. In this section, we focus on the NF-κB-dependent and NF-κB-independent regulation of two types of CAP that are prevalent in the mammalian gut, namely the defensins and cathelicidins. Furthermore, we review the pathological role of these peptides in the development of intestinal inflammation.

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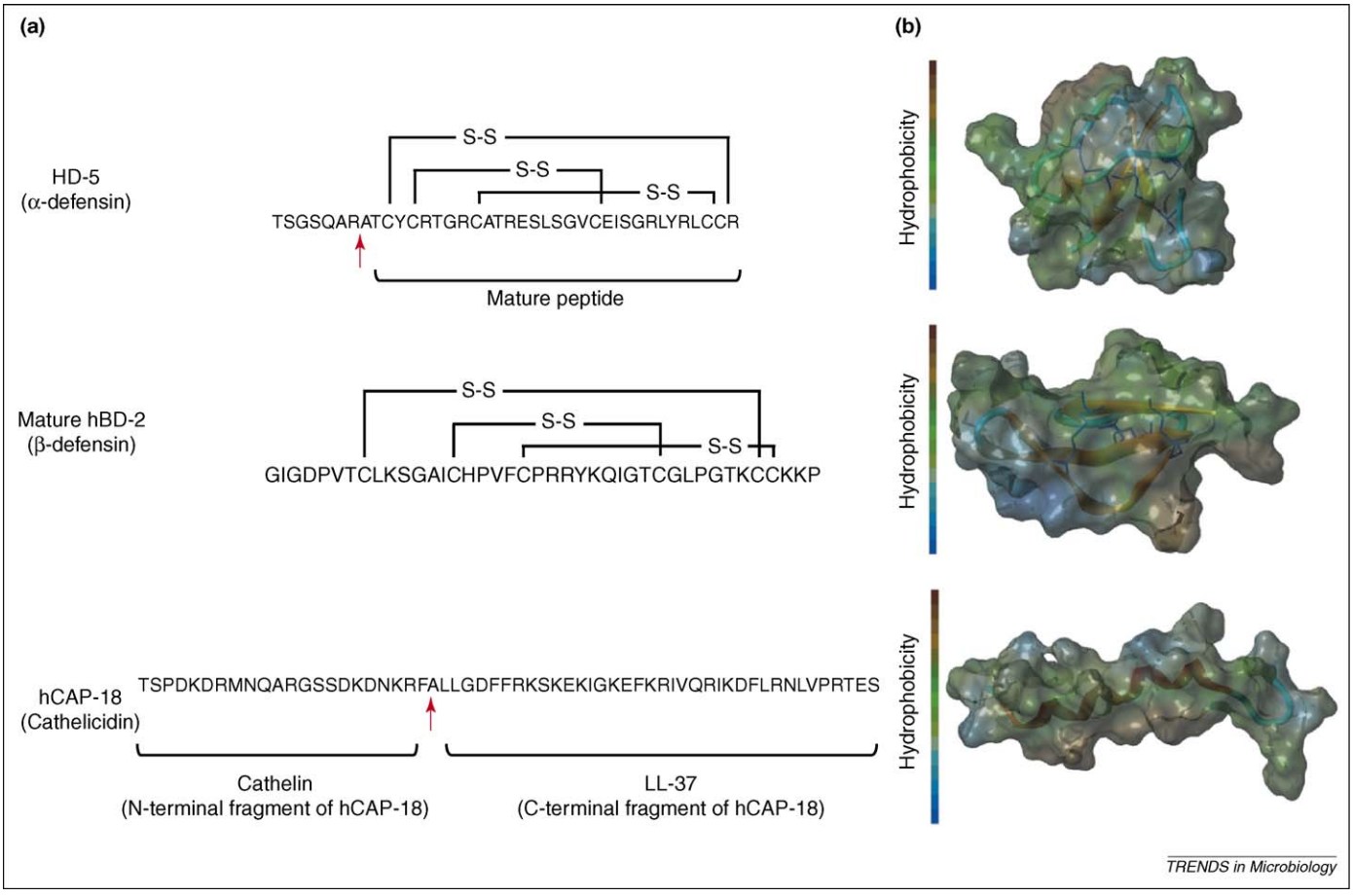


**Figure 1**. Structure of human defensins and cathelicidin. **(a)** Human sequences of the enteric α-defensin HD-5, the β-defensin hBD-2 and the cathelicidin hCAP-18. Red arrows depict the cleavage site for HD-5 and cathelicidin. Patterns of the three intramolecular disulfide bonds of the α- and β-defensins are indicated by black lines (S-S). **(b)** 3D solution (hBD-2 and cathelicidin) and crystal structure (HD-5) of defensins and cathelicidin. Blue sticks within the 3D structures indicate the three intramolecular disulfide bonds. The Protein Data Bank accession numbers used for the illustration are: HD-5, 1ZMP; hBD-2, 1E4Q; and LL-37 (C-terminal fragment of hCAP-18), 2FCG. β turns are in orange and α-helices are in red; molecule hydrophobicity is also displayed.

### *Defensins and cathelicidins*

The α- and β-defensins are small polypeptides with spatially separated hydrophobic and positively charged residues. Six invariant cysteines form three specific intramolecular disulfide bonds and, thus, stabilize the protein in an intricately folded, triple-stranded β-sheet configuration [12,13] (Figure 1). Whereas the α-defensins HD-1 to HD-4 (also known as human neutrophil proteins 1–4) are expressed by neutrophils, HD-5 and HD-6 (also known as cryptdins in mice) are produced by specialized intestinal epithelial cells called Paneth cells. These cells are located primarily at the base of the crypts of Lieberkühn in the small intestine and have a major role in the innate immunity of the ileal mucosa by synthesizing and releasing proteinaceous granules into the lumen following exposure to microbes and/or microbial products. These secretory granules are rich in amphipathic peptides, which can cause microbial death by disrupting membrane integrity [12].

The *in vivo* antimicrobial function of α-defensins has been experimentally exemplified by observation of greater resistance to *Salmonella typhimurium* infection in HD-5 transgenic mice, accompanied by marked changes in the composition of the dominant flora in the gastrointestinal lumen [14]. Unlike β-defensins, the α-defensins produced by Paneth cells are mainly regulated at a post-transcriptional level by extracellular proteases [15], including matrix metalloproteinase-7 (MMP-7, matrilysin) in mice and trypsin in humans [16,17]. Hence, *Mmp-7*$^{-/-}$ mice accumulate inactive forms of cryptdins and succumb more readily to oral infection with *S. typhimurium* than wild-type animals [17]. Six human β-defensins (hBD-1 to hBD-6) are primarily synthesized by most epithelial cells. Mice lacking the *hBD1* orthologue show increased susceptibility to *Staphylococcus aureus* infection [18], which supports a role for this protein in innate immunity.

Cathelicidins are CAPs that are structurally and evolutionary distinct from defensins but have a similar abundance and distribution in the gastrointestinal tract [12]. They are synthesized as large precursor peptides containing a highly conserved N-terminal domain (cathelin), linked to a C-terminal peptide with antimicrobial activity (Figure 1). As with defensins, cathelicidins are activated by extracellular partial proteolysis [19]. Although several members of the family have been identified in other mammalian species, humans and mice have a single cathelicidin gene [referred to as LL-37/FALL39/hCAP18 and cathelin-related anti-microbial peptide (CRAMP), respectively [20]]. Experimental evidence has indicated that mice lacking CRAMP are more susceptible to cutaneous infection by group A streptococci and urinary tract infection by invasive *Escherichia coli* [21,22]. Furthermore,

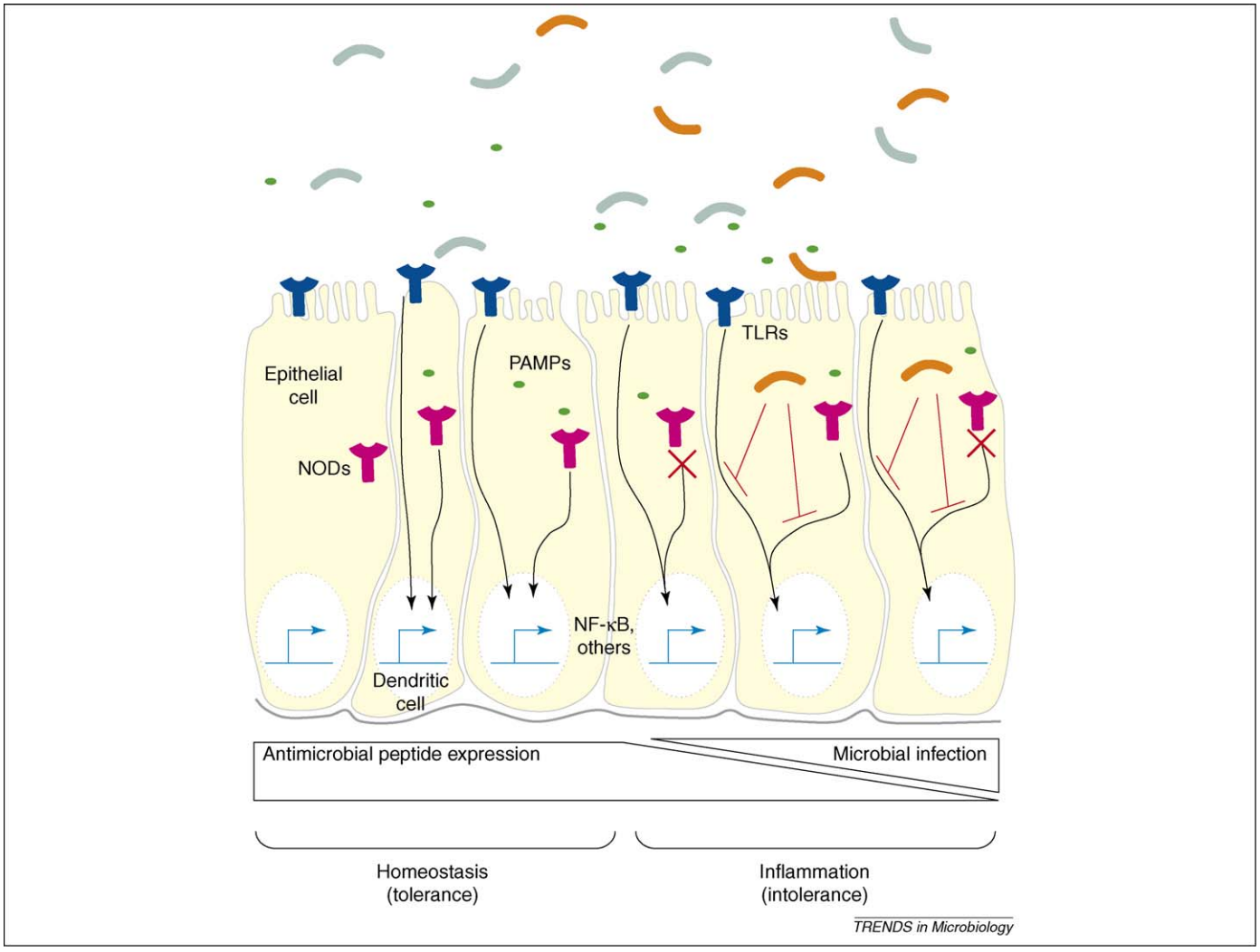


**Figure 2**. A pathophysiological model for chronic intestinal inflammation. Once indigenous (pale blue) or enteroinvasive (orange) microbes and/or their products [pathogen-associated molecular patterns (PAMPs; green ovals)] are sensed by TLRs (dark blue) and/or NODs (pink; left), CAPs are synthesized by the action of NF-κB and/or other transcription factors. Following their secretion and extracellular processing, the CAPs (i) promote tolerance and the recruitment of inflammatory cells; (ii) prevent invasion of microbial pathogens; and (iii) protect against the development of chronic intestinal inflammation. Abnormal antimicrobial peptide synthesis and/or function might lead to aberrant activation of the adaptive immune system and to intestinal inflammation (right) by microbial threats and/or impaired innate immunity (i.e. *NOD2* mutations).

CRAMP-deficient macrophages failed to control replication of *S. typhimurium* [23].

### *NF-κB-dependent and -independent regulation of gut-derived antimicrobial peptides*

Mice that bear mutations in the NF-κB and MyD88 signalling pathways display increased susceptibility to *Helicobacter*-induced colitis [24] and commensal-triggered colitis, respectively [25], which indicates an essential role for NF-κB in gut tolerance and/or resistance to bacteria (Figure 2) and a potential involvement in the regulation of CAP production. In humans, *hBD-1* expression is constitutive in the small intestine and the colon, whereas colonic synthesis of hBD-2–4 is strongly dependent on NF-κB activation by infectious agents in the digestive tract (such as *H. pylori*) and/or pro-inflammatory cytokines [12]. In addition to the regulatory impact of TLR signalling [26], the NOD1 and NOD2 signalling pathways have been shown to trigger *hBD-2* expression [11,27]. Furthermore, recent findings have shown that activation of the MAP kinase pathways is also required for *hBD-2* and/or *-3* expression [11,26].

In parallel, NF-κB-independent signalling pathways might control CAP production by regulating epithelial cell renewal, differentiation and/or lineage commitment. Interestingly, impaired Wingless (Wnt) signalling is associated with a complete lack of proliferative cells in the foetal small intestinal epithelium [28], which suggests that this pathway has an essential role in maintaining the status of intestinal epithelial cells as either proliferative or undifferentiated. The absence of the gene encoding ephrin B3 (which is downregulated by the Wnt signalling pathway) resulted in abnormal Paneth cell lineage commitment [29]. Moreover, the Wnt signalling pathway might monitor defensin gene expression [through transcription factor (TCF)-4] in cells derived from Paneth cells because cryptdins were not detected in the small intestine of embryonic $Tcf4^{-/-}$ or adult mice carrying a conditional deletion of the Wnt receptor Frizzled-5 [30]. Conversely, cryptdin genes are overexpressed in mice that show mutational activation of the Wnt signalling pathway [30,31]. Lastly, a site-directed mutational analysis of α-defensin promoters revealed an essential regulatory role for TCF binding sites

[30]. Taken as a whole, these findings indicate that activation of Wnt signalling is required for the production of Paneth-cell-derived CAPs. Hence, Paneth cell determinants (such as Mtgr1 and Gfi1) should be considered as potential candidates for susceptibility to chronic inflammatory disorders [32,33].

Given the crucial role of certain nuclear receptors in immunity, bacterial-induced inflammation and cell proliferation and maturation, it was suggested (by us and others) that these receptors could have a potential role in gastrointestinal innate immunity by regulating CAP biogenesis. Interestingly, a glucocorticoid receptor agonist (dexamethasone) enhances *hBD-2* expression, although the mechanism remains to be determined [34]. More recently, cathelicidin- and hBD2-encoding genes were identified as targets of the vitamin D receptor (VDR) [35], a nuclear receptor that is required for resistance to *Mycobacterium bovis* infection [36]. Treatment of monocytes with a synthetic VDR ligand led to dose-dependent upregulation of cathelicidin gene transcription, which exerts a direct antimicrobial effect on *Mycobacterium tuberculosis* [37]. In agreement with these findings, individuals with decreased endogenous VDR ligand levels display increased susceptibility to *M. tuberculosis* infection [37]. Furthermore, Shah *et al.* [38] unravelled crosstalk between the Wnt/β-catenin and VDR signalling pathways; further investigation of such phenomena might shed light on the understanding of antimicrobial host defence and could lead to the development of promising therapeutic strategies for chronic inflammatory diseases.

## Hide and seek: when enteric microbes and defensins enter into combat

To circumvent the microbicidal activity of CAPs, microorganisms (generally pathogens) have developed a range of strategies that are reminiscent of those involved in antibiotic resistance [39]. One way to achieve inactivation is to produce proteases, which degrade CAPs; however, in the case of defensins, the intramolecular disulphide bridges render the peptides relatively resistant to enzymatic proteolysis. Another stratagem reduces the net cationic charge of the bacterial envelope to lower its affinity for CAPs: this is achieved by incorporating positively charged groups into the teichoic acid polymers (D-alanine) and into the lipid A (aminoarabinose) in the bacterial cell wall. Other bacterial approaches to CAP resistance include preventing the host effectors from accessing their target through extracellular capture by secretory proteins and actively pumping the peptides across the cytoplasmic membrane [39]. However, despite these various protective weapons (which are not mutually exclusive), microorganisms can probably still be inhibited by CAPs if the host is capable of releasing high amounts of CAPs into the intestinal lumen, as in the case of α-defensins. In such a situation, downregulation of CAP-encoding genes at the transcriptional level by bacterial components (as reported in patients with shigellosis [40] and in mice orally infected with *S. typhimurium* [41]) might be a highly sophisticated counter-mechanism (Figure 2). Additional investigation is now needed to specify the molecular mechanisms by which microbial threats might modulate innate immunity in general and the NOD signalling pathways in particular, and the mechanisms by which enteric microbes might trigger chronic inflammation of the CAP-defective gut.

## Host failure to monitor defensins associated with Crohn's disease

Three mutations in the *NOD2* gene (namely Arg702Trp, Gly908Arg and the frameshift mutation 1007fs) lead to a predisposition to CD [3,4]. Genotype–phenotype correlations have established that NOD2 mutants are predominantly linked to ileal CD [42]. Both common and rare mutations have been associated with impaired MDP-induced NF-κB activation [5,7] and cytokine production in peripheral blood monocytes [7,43–45]. Lala and collaborators recently reported that *NOD2* is highly expressed in Paneth cells [46,47], a finding that might account for the association between *NOD2* mutations and the development of ileal inflammatory lesions [42]. In agreement with a protective effect of NOD2 in the ileum, Nod2-knockout mice displayed an enhanced susceptibility to oral infection (but not systemic infection) with the Gram-positive facultative intracellular bacterium *L. monocytogenes*, and a markedly decreased expression of a subgroup of cryptdin genes [8] (Figure 2).

Importantly, decreased production of HD-5 and HD-6 has been found in surgical resection specimens and biopsies from ileal CD patients [14,48,49]; reportedly, the CD-associated *NOD2* mutations contributed to this impairment. By contrast, individuals with Crohn's colitis displayed normal α-defensin levels but have a much-reduced copy number for the β-defensin gene *hBD-2*, resulting in impaired expression in the colon [50]. As with *NOD2* mutations, complex intronic polymorphism of the *NOD1* gene has been associated with the pathogenesis of IBD [51]. In addition, Nod1-deficient mice display increased susceptibility to *H. pylori* infection [52] and decreased expression of certain β-defensins [11]. Taken as a whole, these observations suggest an overall pathophysiological concept for CD (Figure 2). Complementary studies are now required to specify the molecular link between NOD1/2 and α/β-defensin expression and to determine the spectrum of oral susceptibility to other microbes in Nod-deficient mice. Finally, the role of other NOD-dependent signalling pathways in CAP expression remains to be determined. It also remains to be seen whether CAP-deficient mice experience spontaneous and/or increased susceptibility to experimentally induced colitis (Box 1).

**Box 1. Questions for future research**

- Does the commensal flora have a regulatory role in CAP expression?
- What is the spectrum of action of CAPs on indigenous and pathogenic bacteria?
- How is the diversity of the commensal flora regulated by CAPs?
- Which TLRs and NODs are implicated in defensin regulation?
- How do CAPs alert the host immune system to the presence of intruders?
- Is there any synergy and/or redundancy between TLRs and NODs in terms of CAP biogenesis?

**Table 1. Versatile functions of the defensins and cathelicidins[a]**

| Functions | Enteric α-defensins | β-defensins | Cathelicidin | Refs |
|---|---|---|---|---|
| **Innate immunity** | | | | |
| Antimicrobial | Yes | Yes | Yes | [12,13,21,39,53] |
| Endotoxin binding | ND | ND | Yes | [39,53] |
| Phagocyte chemotaxis | ND | Yes | Yes | [12,13,53] |
| Cytokine production | Yes | Yes | Yes | [54–56] |
| Chemokine production | Yes | ND | Yes | [53,54] |
| Mast cell degranulation | ND | Yes | Yes | [13,53] |
| **Adaptive immunity** | | | | |
| Dendritic cell activation and/or maturation | ND | Yes | Yes | [54–56] |
| Dendritic cell chemotaxis | ND | Yes | ND | [12,13,53] |
| T-lymphocyte activation | ND | Yes | Yes | [56] |
| T-lymphocyte chemotaxis | ND | Yes | Yes | [12,13,53] |
| Immunoglobulin production | ND | Yes | Yes | [53] |
| **Others** | | | | |
| Angiogenic | ND | Yes | Yes | [13,57] |
| Apoptotic | ND | ND | Yes[b] | [53] |
| Anti-tumoral or cytotoxic | ND | Yes | Yes[c] | [12,53] |
| Hydroelectrolyte secretion by enterocytes | Yes | ND | ND | [12,53] |

[a]Abbreviation: ND, not determined.
[b]Inhibition of neutrophil apoptosis or induction of epithelial cell apoptosis.
[c]At high concentrations.

## Gastrointestinal antimicrobial peptides: multifaceted molecules

The antimicrobial activity of CAPs seems to be only the 'tip of the iceberg' because pleiotropic functions have been attributed to defensins and cathelicidins [53] (Table 1). Both CAPs have the ability to chemoattract immunocytes involved in innate immunity (neutrophils and monocytes or macrophages), adaptive immunity (dendritic cells and T lymphocytes) and allergic or inflammatory reactions (mast cells). Furthermore, hBD-2 might activate the TLR4-dependent signalling pathway in dendritic cells [54]. By contrast, Hancock and colleagues recently reported that LL-37 might dampen TLR-dependent activation in human monocytes [55] and could promote maturation of dendritic cells, resulting in Th1 polarization of T cells [56]. Taken as a whole, these findings indicate that CAP interactions might initiate and control the inflammatory response by linking innate and acquired immunity (Table 1). Finally, CAPs such as LL-37 have the ability to promote angiogenesis, as demonstrated by decreased vascularization during skin wound repair in CRAMP-deficient mice [57]. Because Paneth cell biology influences intestinal angiogenesis through the recognition of commensals [58], these findings might provide new insight into the pathogenesis of IBD.

## Concluding remarks

Enteric CAPs have several essential and emerging roles in both the innate and adaptive immunity of the gastrointestinal tract by modulating microbial resistance, angiogenesis and chemotaxis and by promoting the humoral response (Table 1). In particular, release of CAPs into the lumen is thought to protect the mitotically active crypt cells (which renew the epithelial cell monolayer) from colonization by pathogenic microbes. The use of transgenic animals would yield a better understanding of the physiological role and regulation of these effectors. NOD1 and -2 have been shown to exert bactericidal activity by modulating the epithelial production of defensins, which suggests a possible mechanism whereby PRMs might protect the host from the development of CD (Figure 2). Furthermore, reduced expression of defensins in ileal CD might contribute to changes in the luminal flora, thus generating vulnerability throughout the epithelial barrier to infection with CD-associated pathogens such as adherent-invasive *E. coli* [59] and *Mycobacterium paratuberculosis* [60] (Figure 2). The influence of other microbial sensors and the CAPs on the emergence of colonic disease also needs to be clarified because the physiological bacterial load is higher in the colon than in the small intestine. Finally, the use of mice with mutant CAPs and a recently developed mouse model carrying the major CD-associated *NOD2* mutation [61] might help to determine whether impaired enteric defensin function and/or natural NOD mutations are sufficient to trigger the development of intestinal inflammatory diseases.

In conclusion, we believe that the development of novel therapeutic strategies should now focus on tackling the abnormal CAP expression observed in the gastrointestinal tract of susceptible individuals; this could include the use of synthetic CAP-like molecules (intrabiotic therapy) [62] and/or modulators of CAP expression. In particular, the increased β-defensin expression in inflammatory lesions of patients with ulcerative colitis [63,64] could be diminished by synbiotic therapy (a prebiotic–probiotic combination) [65]. It is also noteworthy that *hBD-2* expression can be induced by certain probiotic strains [66] (such as *E. coli* Nissle 1917), which suggests that abnormal defensin expression might be improved by rational therapy.

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